

GROWTH AND DIFFERENTIATION OF HUMAN OSTEOBLAST-LIKE CELLS ON Ti-6Al-4V ALLOY MODIFIED WITH ANODIZATION

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Abstract

Titanium and its alloys are widely used as hard tissue implants for their high biocompatibility and suitable physical properties. Along with other surface treatment methods, anodization technique is known to enhance osseointegration. The aim of this study is to evaluate the adhesion, growth and osteogenic differentiation of human osteoblast-like SAOS-2 cells on Ti-6Al-4V samples anodized in electrolytes composed of a stable volume of KOH (336.48 g/l) and variable volumes of liquid glass (124.47 g/l, 84,12 g/l and 37.38 g/l; samples s5, s6 and s7, respectively). Non-anodized Ti-6Al-4V samples, cell culture polystyrene (PS) and microscopic glass coverslips served as control materials. On days 2 and 4 after seeding, the cell number did not differ significantly among the tested samples. However, on day 7, the cell number on s6 samples reached the lowest values, which could be attributed to a non-homogeneous TiO₂ film on s6 samples formed during anodization. Nevertheless, the osteogenic differentiation, estimated by the intensity of fluorescence of collagen I in cells grown in a differentiation medium, was the highest on s6 samples. On s5 samples, coated with homogeneous TiO₂ films, both cell numbers and intensity of fluorescence of collagen I was relatively high. The bone matrix mineralization, evaluated by Alizarin Red staining, was the highest on s5 samples in standard culture medium, and similar on all tested samples in differentiation medium. Thus, the surface modification of s5 samples could be considered the most suitable for application in bone implants.

Keywords: Ti6-Al4-V, SAOS-2 cells, anodization, bone implants, collagen I, mineralization

1. INTRODUCTION

Biomaterials such as titanium (Ti) and its alloys or compounds (e.g., TiO₂) have various medical applications (from implants to drug delivery systems) and are extensively used especially in the fields of orthopedics and dental medicine. For use in hard tissue implants (aside from its favourable mechanical properties and biocompatibility) the materials promoting a better integration with the surrounding bone tissue are desirable, thus the improved adhesion, proliferation and osteogenic differentiation of cells on these surfaces is very important for designing and fabricating the implants. Properties such as surface chemistry, morphology and structure of these materials are important factors to influence the cell behaviour. Many techniques are used to modify the titanium implant surfaces (for a review see [1]), e.g. mechanical methods to alter the surface morphology [2-4], and also various biochemical methods using inorganic compounds and biomolecules [5-7].

In this article we tested the response of human osteoblast-like SAOS-2 cells to Ti6-Al4-V alloy samples modified by an anodization technique to form a TiO₂ (i.e., anatase or rutile) film on their surface. Ti oxide layers are known to improve the corrosion resistance of the bulk material. Moreover it has been observed that an anatase crystalline form of TiO₂ exhibit antimicrobial effects [8] and may play a positive role in the osseointegration process thanks to its effects on calcium phosphate precipitation [9].

2. MATERIALS AND METHODS

2.1. Metallic sample preparation and characterization

Series of Ti-6Al-4V alloy samples were prepared in the form of disks with 14 mm in diameter and 2 mm in thickness. Surfaces of some samples were further modified with anodization (samples s5, s6, s7) in electrolytes of a different ratio composition of KOH and liquid glass (**Table 1**), while samples sC (control Ti-6Al-4V sample group) were left unmodified. Ten samples per each experimental group were used. Anodization was performed using a voltage of 40 V for 60 seconds to form a coating layer of TiO₂.

The chemical composition and morphology of the surfaces of all anodized samples were observed and measured by means of confocal Raman microscopy (XploRA™, HORIBA Jobin Yvon, France) coupled with an optical microscope (Olympus BX41/51, obj. 100x) and 532 nm wave-length laser (20-25 mW, 3B grade).

Table 1 Electrolyte composition for surface treatment

Sample	KOH [g/l]	Liquid glass [g/l]
s5	336.48	124.48
s6	336.48	84.12
s7	336.48	37.38

2.2. Cell culture on metallic samples

The Ti-6Al-4V samples (s5, s6, s7, and sC) were cleansed in 70% ethanol, rinsed with distilled and deionized water, sterilized in an autoclave (120 °C, 1 atm., 30 min) and air-dried in a laminar flow box under sterile conditions. The samples were inserted into 24-well polystyrene cell culture plates (TPP, Trasadingen, Switzerland; well diameter 15 mm) and seeded with human osteoblast-like SAOS-2 cells (ATCC-HTB-85, Chemos) in an initial density of 20,000 cells per well (approx. 11,300 cells / cm²). Polystyrene cell culture wells (PS) and microscopic glass coverslips (sGI) inserted in these wells were used as reference materials. Cultures were supplied with 1.5 ml of McCoy's 5A medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS, GIBCO, Life Technologies) and 40 µg/mL of gentamicin (Gentamicin Lek, Ljubljana, Slovenia), referred as "growth medium". The cells were cultured in a thermostat at 37 °C in a humidified atmosphere containing 95 % of air and 5 % of CO₂. On days 2, 4 and 7 after seeding, the cells were rinsed in phosphate-buffered saline (PBS), fixed with 70% cold ethanol and stained by Texas Red C₂-maleimide (20 ng/mL) and Hoechst # 333258 (5 µg/mL) (both Sigma-Aldrich Co.) in PBS for 20 minutes. These samples were evaluated for the cell population density and proliferation by fluorescence microscopy (IX 51 epifluorescence microscope equipped with a DP 70 digital camera, Olympus Corp., Tokyo, Japan). For each experimental group and time interval two samples were used. From each sample 10 images of random areas were acquired and visible cell nuclei were manually counted.

On day 7 after seeding, one half of the samples was supplied with differentiation medium, i.e. the growth medium mentioned above further supplemented with 10 mM β-glycerolphosphate, 2 mM L-glutamine, 50 µg/ml of ascorbic acid, 10⁻⁶ M dihydroxyvitamin D₃ and 10⁻⁸ M dexamethasone. The second half of samples was supplied with fresh growth medium. The cells were cultured for additional 9 days.

The production of collagen type I, i.e. early marker of osteogenic cell differentiation, was evaluated using immunofluorescence staining and subsequent image analysis. The cells were rinsed with PBS and fixed with 70% cold ethanol. Non-specific binding sites were then blocked with 1% bovine serum albumin supplemented with 0.1% triton in PBS for 20 minutes, and then with 1% Tween for additional 20 minutes. The primary antibody, i.e. rabbit anti type I collagen antibody (Cosmo Bio Co., Ltd.), was diluted to the ratio of 1:200 in PBS and applied overnight at 4 °C. After rinsing, the cells were incubated for one hour at room temperature with the secondary antibody, i.e. Alexa Fluor 488®-conjugated F(ab') fragment of goat anti-rabbit IgG (H+L), (Molecular Probes, Cat. No. A11070), diluted 1:400 in PBS with Hoechst # 333258 (5 µg/mL, Sigma-

Aldrich Co.) in order to stain the cell nuclei. Images of 10 random areas from each sample were acquired by fluorescence microscope (Olympus IX 51, digital camera DP70, Japan) at the same exposure time using two different filters for each area (one visualizing cell nuclei and the other visualizing collagen). The fluorescence intensity was measured using Fluorescent Image Analysis software (ALICE, version 1.0) [10] and normalized for the number of cells. The fluorescence intensity of the samples stained without primary antibodies was subtracted.

The formation of mineralized matrix, i.e. another indicator of osteogenic cell differentiation, by SAOS-2 cells on tested samples was evaluated by staining with Alizarin Red S (Sigma Aldrich, USA). After staining, the cells with mineral deposits were detached from the sample surface by 10% acetic acid, and the absorbance was measured spectrophotometrically using a Versa Max Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA.) at 405 nm wave-length.

3. RESULTS AND DISCUSSION

3.1. Morphology and chemical composition of the sample surface

Surface morphology of the anodized samples is displayed in **Fig. 1**. It is apparent that after anodization, the samples contain irregularities on their surfaces, while the control non-anodized Ti-6Al-4V alloy were rather smooth (sample not shown).

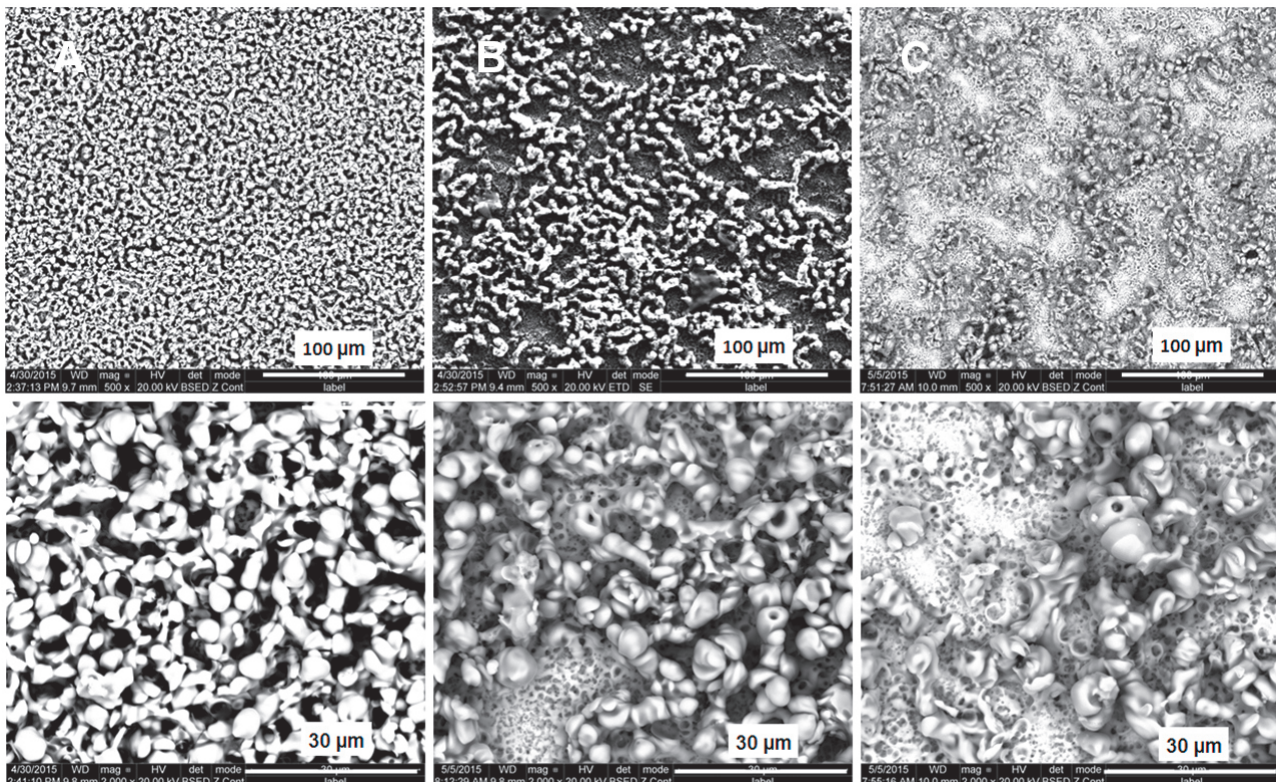


Fig. 1 Surface morphology of s5, s6 and s7 samples (A, B, C respectively). First row: overall view, second row: detailed view

Raman spectroscopy revealed that the surface layer of anodized samples is composed of TiO₂, predominantly in the anatase crystalline form, although the presence of rutile structures has not been ruled out. Homogenous TiO₂ layer was formed in the electrolyte used for s5 samples. The layers on samples s6 and s7 were increasingly less homogenous. Accordingly, the surface roughness of these samples reached higher values (R_a 0.89 μm, 1.54 μm and 2.28 μm in samples s5, s6 and s7, respectively).

3.2. Adhesion, growth and osteogenic differentiation of SAOS-2 cells

On days 2 and 4 after seeding, the cell number in the growth medium was similar among the tested samples (**Fig. 2A**). However, on day 7, the cell number on s6 samples reached the lowest values, which could be attributed to a less homogeneous TiO₂ film on these samples. It is known that TiO₂, anatase in particular, support the cell adhesion and growth, especially by its enhanced wettability (for a review, see [11]). The cells on all tested samples on day 7 were well-spread and polygonal, but they formed confluent or at least subconfluent layers only on control unmodified T-6Al-4V (sC) samples (**Fig. 3**).

The cell number on s6 samples also remained low after additional 9 days of cultivation in the medium promoting osteogenic cell differentiation (**Fig. 2B**). It is known that if the cells are stimulated to differentiate, they attenuate their proliferation activity (for a review, see [12]), which was apparent on s6 samples and control unmodified (sC) samples. On the other samples, the cell numbers in the growth and differentiation media were similar, and on s5 sample, the cell number in the differentiation medium was even higher.

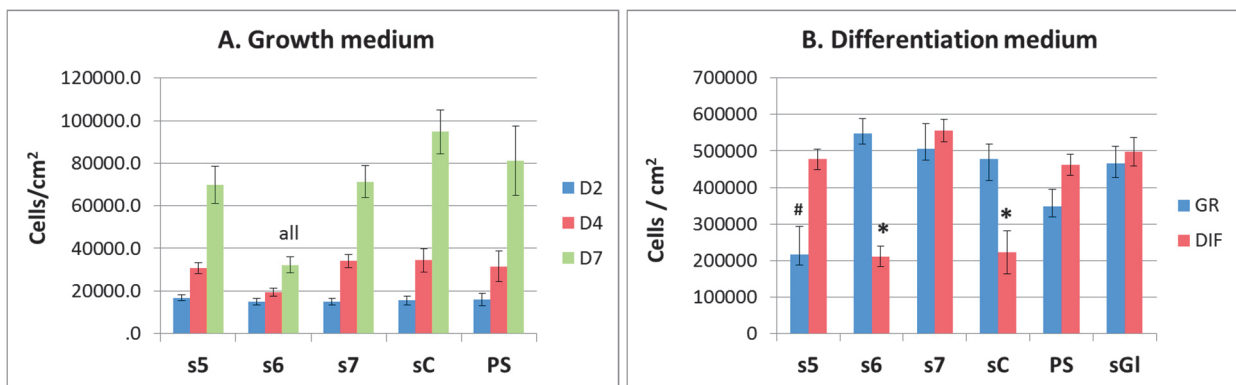


Fig. 2 Number of SAOS-2 cells on s5, s6 and s7 samples, control untreated samples (sC), polystyrene wells (PS) and glass coverslips (sGI) on days 2,4 and 7 after seeding (D2, D4, D7) in the growth medium (A) and after additional 9 days of cultivation in growth (GR) or differentiation (DIF) media (B). Mean S.E.M. from 20 measurements, ANOVA, Student-Newman-Keuls method. Statistical significance ($p \leq 0.05$): all: compared to all other samples, *: compared to s5, s7, PS and sGI, #: compared to all samples except PS

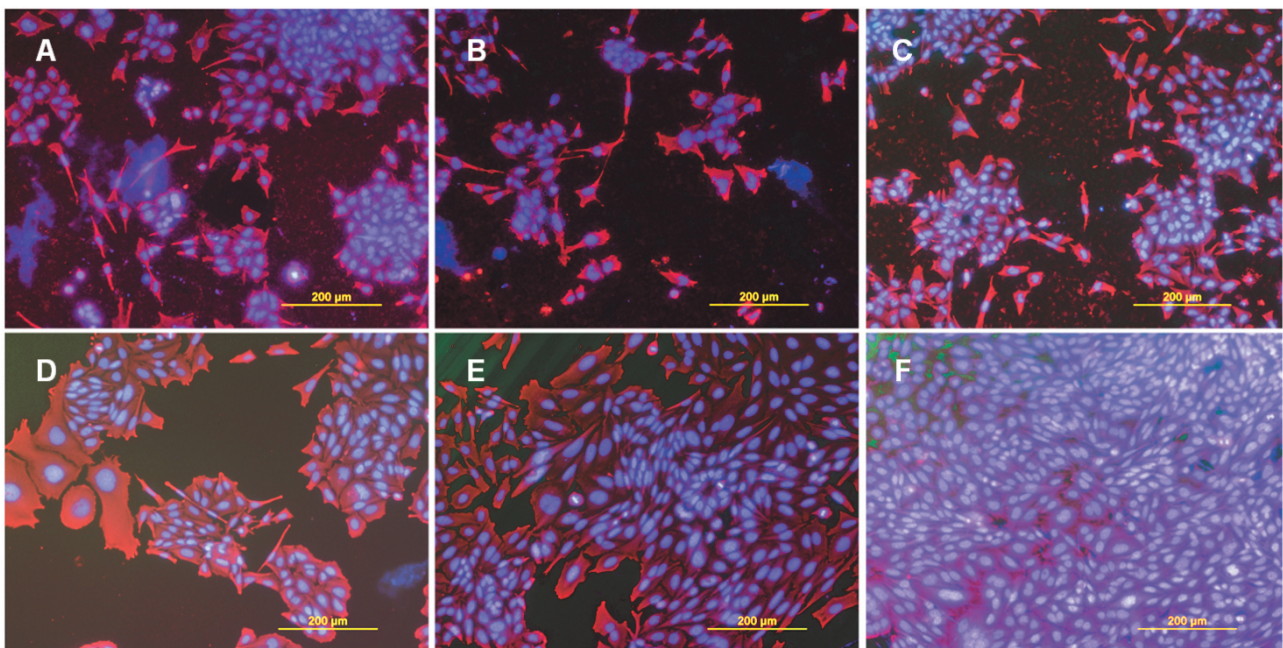


Fig. 3 Morphology of SAOS-2 cells on day 7 after seeding on s5, s6, s7 samples (A, B, C), and on control PS and two control samples sC1, sC2 (D, E, F)

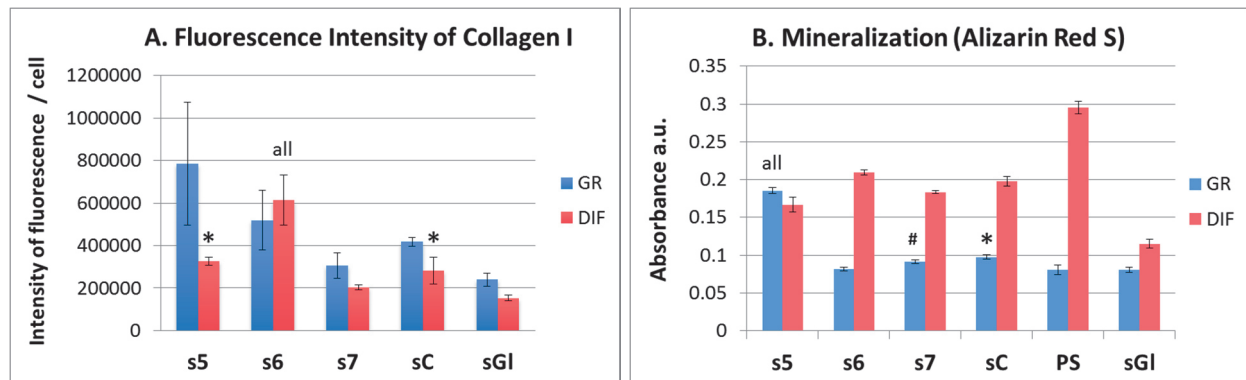


Fig. 4 Osteogenic cell differentiation measured by the intensity of fluorescence of collagen I (A) and mineralization (B) after 9 days of SAOS-2 cell cultivation on s5, s6 and s7 samples, control untreated samples (sC), polystyrene wells (PS) and glass coverslips (sGI) in growth (GR) or differentiation (DIF) media. Mean S.E.M. from 10 measurements, ANOVA, Student-Newman-Keuls method. Statistical significance ($p \leq 0.05$): all: compared to all other samples, *: compared to s6, s7, PS and sGI, #: compared to s6, sGI and PS

4. CONCLUSION

It can be concluded that the SAOS-2 cells cultured on s5 samples reached relatively high numbers and showed a relatively high intensity of fluorescence of collagen I, an early marker of osteogenic cell differentiation. At the same time, the bone matrix mineralization, evaluated by Alizarin Red S staining, was the highest on these samples. Thus, the mode of anodization applied for s5 samples seems to be the most advantageous for the surface modification of bone implants.

ACKNOWLEDGEMENTS

Supported by the Technological Agency of the Czech Republic (TACR grant No. TA04011214) and the Ministry of Health of the CR (grant No. 15-32497A).

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